

Research Article

Extracellular Calcium-Dependent Modulation of Endothelium Relaxation in Rat Mesenteric Small Artery: The Role of Potassium Signaling

Lise Hangaard,¹ Peter B. Jessen,¹ Dmitrii Kamaev,¹
Christian Aalkjaer,^{1,2} and Vladimir V. Matchkov¹

¹MEMBRANES, Department of Biomedicine, Health, Aarhus University, 8000 Aarhus, Denmark

²Department of Biomedicine, University of Copenhagen, Copenhagen, Denmark

Correspondence should be addressed to Vladimir V. Matchkov; vvm@fi.au.dk

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The nature of NO- and COX-independent endothelial hyperpolarization (EDH) is not fully understood but activation of small- and intermittent-conductance Ca^{2+} -activated K^+ channels (SK_{Ca} and IK_{Ca}) is important. Previous studies have suggested that the significance of IK_{Ca} depends on $[\text{Ca}^{2+}]_{\text{out}}$. Also it has been suggested that K^+ is important through localized $[\text{K}^+]_{\text{out}}$ signaling causing activation of the Na^+ , K^+ -ATPase and inward-rectifying K^+ channels (K_{ir}). Here we tested the hypothesis that the modulating effect of $[\text{Ca}^{2+}]_{\text{out}}$ on the EDH-like response depends on $[\text{K}^+]_{\text{out}}$. We addressed this possibility using isometric myography of rat mesenteric small arteries. When $[\text{K}^+]_{\text{out}}$ was 4.2 mM, relaxation to acetylcholine (ACh) was stronger at 2.5 mM $[\text{Ca}^{2+}]_{\text{out}}$ than at 1 mM $[\text{Ca}^{2+}]_{\text{out}}$. Inhibition of IK_{Ca} with TRAM34 suppressed the relaxations but did not change the relation between the relaxations at the low and high $[\text{Ca}^{2+}]_{\text{out}}$. This $[\text{Ca}^{2+}]_{\text{out}}$ -dependence disappeared at 5.9 mM $[\text{K}^+]_{\text{out}}$ and in the presence of ouabain or BaCl_2 . Our results suggest that IK_{Ca} are involved in the localized $[\text{K}^+]_{\text{out}}$ signaling which acts through the Na^+ , K^+ -ATPase and K_{ir} channels and that the significance of this endothelium-dependent pathway is modulated by $[\text{Ca}^{2+}]_{\text{out}}$.

1. Introduction

The importance of the arterial endothelium for controlling vascular resistance is well-established [1]. Several factors are released from the endothelium and relax the adjacent smooth muscle cells. In addition to NO and prostaglandins, the endothelium is able to produce vasodilatation through a third pathway, which is particularly important in small arteries and arterioles [2]. The third pathway is the endothelium-dependent hyperpolarization (EDH) of smooth muscles, which is present after the inhibition of NO and prostaglandin production [3, 4]. The mechanisms proposed for EDH are direct spreading of hyperpolarizing current via myoendothelial gap junctions or release of diffusible factor(s) (EDHF(s)). The relative contribution of these two pathways and the nature of the EDHFs vary among species and vascular beds,

blood vessel caliber, ageing, and diseases, as well as between different experimental conditions and laboratories [3, 5]. It is unlikely that a single factor accounts for EDHF and multiple diffusible factors, including K^+ , NO, HNO, epoxyicosatrienoic acids, H_2S , H_2O_2 , and vasoactive peptides, have been suggested.

A key element in the EDH pathway supported by most studies is activation of endothelial small- and intermittent-conductance Ca^{2+} -activated K^+ channels (SK_{Ca} and IK_{Ca} , resp.) upon agonist- or shear stress-induced increase of endothelial cell calcium ($[\text{Ca}^{2+}]_{\text{in}}$) [6]. Although activation of both SK_{Ca} and IK_{Ca} channels can lead to vasodilation, their contribution to the EDH is different [3–5]. SK_{Ca} channels are distributed homogeneously over the endothelial cell membrane and respond to increase of $[\text{Ca}^{2+}]_{\text{in}}$ [7, 8]. Hyperpolarization produced by the SK_{Ca} channels is believed

to spread via myoendothelial gap junctions to smooth muscle cells. This original hypothesis is, however, modified [5, 9] based on the observation that SK_{Ca}-dependent hyperpolarization can be blocked not only by apamin, a SK_{Ca} channel inhibitor, but also by Ba²⁺ in concentrations specifically blocking the inward-rectifying K⁺ channels (K_{ir}) [10]. It has been suggested that K⁺ efflux through the opened SK_{Ca} channels might increase the local [K⁺]_{out} and consequently open K_{ir} channels. In this model the localized [K⁺]_{out} increase amplifies endothelial hyperpolarization generated by SK_{Ca} channel opening [5].

In contrast, the IK_{Ca} channels are localized in endothelial cell projections near the adjacent smooth muscle cells [11] where K⁺ efflux through these channels increases [K⁺]_{out} by about 6 mM [12], which has been called a “K⁺ cloud” [13]. The “K⁺ cloud” may hyperpolarize smooth muscle cells via activation of the Na⁺,K⁺-ATPase and K_{ir} channels [11, 14]. This hypothesis is supported by ouabain- and Ba²⁺-sensitive hyperpolarization of smooth muscles in endothelium-denuded arteries with few mM elevation of [K⁺]_{out} [12]. Both these K⁺ sensors, that is, the Na,K-ATPase and the K_{ir} channels, are expressed in smooth muscle cells [11, 15] although their importance varies between vascular beds [5].

How endothelial excitation leads to differentiated activation of SK_{Ca}/IK_{Ca} channels remains unclear but this may be modulated by [Ca²⁺]_{out} via the Ca²⁺-sensing receptor (CaSR) [16]. Thus, changes in [Ca²⁺]_{out} may switch the EDH signaling between being predominantly SK_{Ca}-/myoendothelial gap junction-dependent and being IK_{Ca}-/“K⁺ cloud” dependent pathways.

Based on these considerations we hypothesized that modulation of the EDH-like response by [Ca²⁺]_{out} is dependent on [K⁺]_{out} and tested the importance of IK_{Ca}, Na⁺,K⁺-ATPase, and K_{ir} channels for these effects of [Ca²⁺]_{out} and [K⁺]_{out}.

2. Methods

All experiments were approved by and conducted with permission from the Animal Experiments Inspectorate of the Danish Ministry of Food, Agriculture and Fisheries. Rats were euthanized by CO₂-inhalation.

In vitro functional experiments were performed on rat mesenteric small artery. Rat mesentery was dissected and placed in ice-cold physiological salt solution (PSS). Third-order branches of the rat mesenteric small artery were dissected. The cleaned arterial segments were mounted in an isometric wire myograph (Danish Myo Technology A/S, Denmark) as described previously [17]. The myograph chamber was heated to 37°C, while the PSS was constantly aerated with 5% CO₂ in air. Force was recorded with a PowerLab 4/25-Chart7 acquisition system (ADInstruments Ltd., New Zealand) and converted to wall tension by dividing force with double segment length. Contractile concentration-response relationships were constructed by cumulative noradrenaline concentrations (NA: 0.1–30 μM). The relaxation concentration-response relationships were constructed by

cumulative addition of acetylcholine (ACh) (0.01–10 μM) to the arteries precontracted with 6 μM noradrenaline. A maximum of three concentration-response relaxations were made on one artery.

The 4.2 mM [K⁺]-containing PSS composition was (in mM) 119.00 NaCl, 3.0 KCl, 1.18 KH₂PO₄, 1.17 MgCl₂, 25.0 NaHCO₃, 0.026 EDTA, and 5.5 glucose, gassed with 5% CO₂ in air and adjusted to pH 7.4. The 5.9 mM [K⁺]-containing PSS composition was (in mM) 119.00 NaCl, 4.7 KCl, 1.18 KH₂PO₄, 1.17 MgSO₄, 25.0 NaHCO₃, 0.026 EDTA, and 5.5 glucose, gassed with 5% CO₂ in air and adjusted to pH 7.4. Either 1 mM or 2.5 mM CaCl₂ was added to the PSS as indicated. All chemicals were obtained from Sigma-Aldrich (Brøndby, Denmark). Drugs were applied 15 minutes before experiment.

Statistical analyses were performed using GraphPad Prism 5 (Graph Pad Software Inc., USA). Data are expressed as mean values ± SEM. Concentration-response curves were fitted to experimental data using four-parameter, nonlinear regression curve fitting. From these curves, pD₂ (the concentration required to produce a half-maximal response) and maximal response were derived and compared using an extra sum-of-squares *F* test. *t*-test was used where appropriate. *P* < 0.05 was considered significant. *n* refers to number of rats.

3. Results

The sensitivities (pD₂) and maximal responses to noradrenaline were the same with all four experimental solutions, that is, 1 mM and 2.5 mM [Ca²⁺]_{out} and 4.2 mM or 5.9 mM [K⁺]_{out} (Figure 1(a)). Thus, the precontraction levels in relaxation experiments were the same in all experimental conditions. Inhibition of the IK_{Ca} channels with 10 μM TRAM34 did not affect the concentration-response relationship to noradrenaline (Figure 1(b)). Neither pD₂ nor maximal contractile responses were significantly affected by TRAM34.

Arteries precontracted with 6 μM noradrenaline were relaxed with cumulative addition of ACh (Figure 2). The precontraction remained stable in time-control experiments where only vehicle was applied (Figures 2(a) and 2(b)).

In the 4.2 mM [K⁺]_{out} containing solution the relaxation was more pronounced when [Ca²⁺]_{out} was 2.5 mM in comparison with 1 mM [Ca²⁺]_{out} (Figures 2(a) and 2(c)). This was associated with a higher sensitivity to ACh, pD₂ 7.16 ± 0.07 versus 6.45 ± 0.09 (*P* < 0.01, *n* = 8). When [K⁺]_{out} in the bath solution was elevated to 5.9 mM, no difference between concentration-response curves at 1 mM and 2.5 mM [Ca²⁺]_{out} was observed (Figures 2(b) and 2(d)); pD₂ were 7.06 ± 0.08 and 7.23 ± 0.05 (*n* = 6), respectively.

Preincubation of arteries with 100 μM L-NAME and 3 μM indomethacin significantly suppressed the relaxation to ACh in the presence of both 4.2 mM and 5.9 mM [K⁺]_{out}. Importantly, these inhibitors shifted the concentration-response curves to the right (Figure 2). However, when the bath solution contained 4.2 mM [K⁺]_{out} the relaxation was still more pronounced in the presence of 2.5 mM compared to 1 mM [Ca²⁺]_{out} (Figures 2(a) and 2(c)). When [K⁺]_{out} was 5.9 mM, no difference in the relaxations at different [Ca²⁺]_{out} was seen (Figures 2(b) and 2(d)).

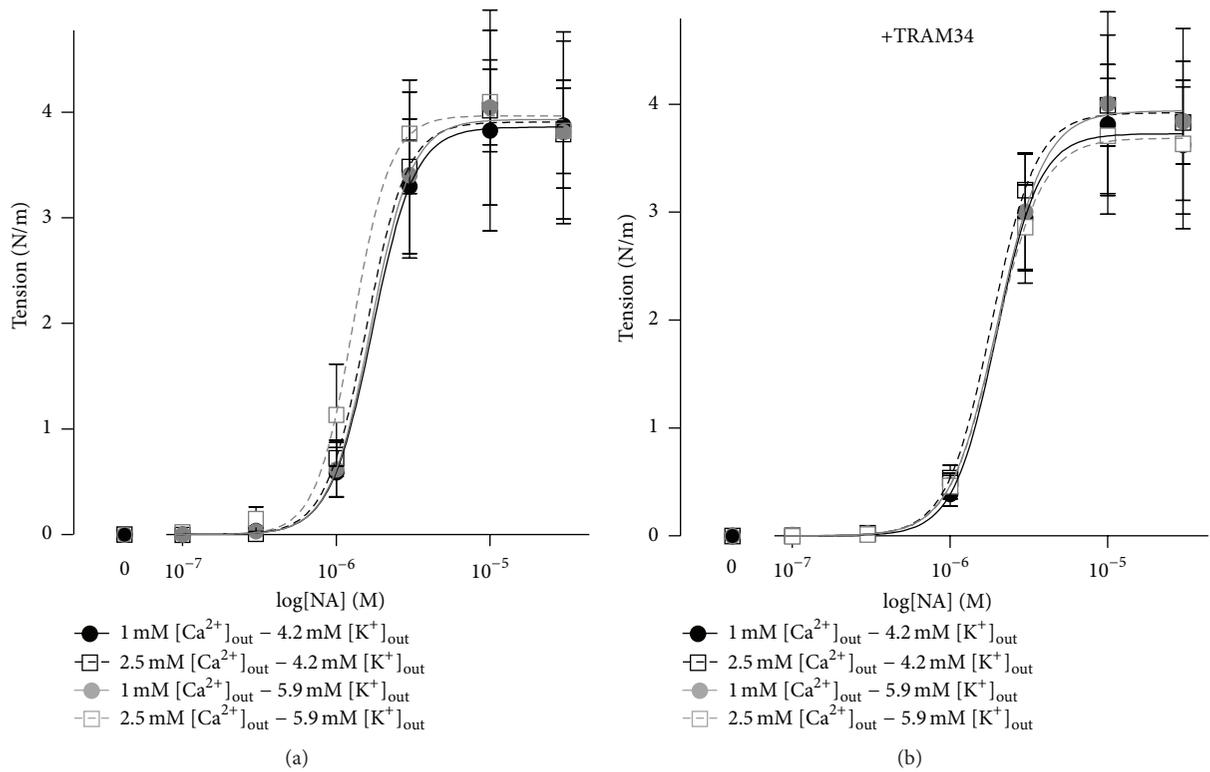


FIGURE 1: Concentration-dependent contractions to noradrenaline (NA) of rat mesenteric small arteries were not different in the presence of 1 mM and 2.5 mM [Ca²⁺]_{out}. The contractions were also the same in solutions with 4.2 mM [K⁺]_{out} and with 5.9 mM [K⁺]_{out}. Preincubation with 10 μM TRAM34 was without significant effect on the contractile responses ((a) versus (b)). *n* = 6.

Repeated relaxations to cumulative addition of ACh in the presence of L-NAME and indomethacin were similar (Figure 3), demonstrating that there was no time-dependent effect on relaxation to ACh.

To assess the importance of I_{KCa} for the effect of [Ca²⁺]_{out} we repeated the experiments in the presence of 1 μM TRAM34. TRAM34 suppressed the relaxations to ACh in the presence of L-NAME and indomethacin (Figures 4(a) and 4(b)). In the 4.2 mM [K⁺]_{out} containing solution the relaxations in the presence of TRAM34 were still stronger at 2.5 mM than at 1 mM [Ca²⁺]_{out}. When [K⁺]_{out} was 5.9 mM no difference in relaxations at 1 mM and 2.5 mM [Ca²⁺]_{out} was seen in the presence of TRAM34 (Figure 4(c)).

In spite of similar changes in the areas under the curve, TRAM34 affected the concentration-response curves differently at 1 mM and 2.5 mM [Ca²⁺]_{out} when [K⁺]_{out} was 4.2 mM. TRAM34 reduced the sensitivity to ACh at 2.5 mM [Ca²⁺]_{out} (Figure 4(d)) but suppressed the maximal relaxation at 1 mM [Ca²⁺]_{out} (Figure 4(e)).

At 5.9 mM [K⁺]_{out} TRAM34 had the same effect on pD₂ to ACh in the presence of 1 mM and 2.5 mM [Ca²⁺]_{out} (Figure 4(d)). No effect of TRAM34 on maximal relaxation was seen in the presence of 5.9 mM [K⁺]_{out} (Figure 4(e)).

Preincubation of arteries with L-NAME, indomethacin, TRAM34, and apamin completely inhibited the ACh-dependent relaxations independently of [K⁺]_{out} and [Ca²⁺]_{out} (not shown, *n* = 6–8).

To assess the importance of the Na⁺,K⁺-ATPase for the effect of [Ca²⁺]_{out} we repeated the experiments in the presence of 10 μM ouabain. Ouabain suppressed the ACh-induced relaxation in the presence of 100 μM L-NAME and 3 μM indomethacin (Figure 5). Ouabain suppressed the relaxation more in the presence of 4.2 mM [K⁺]_{out} than in the presence of 5.9 mM [K⁺]_{out} (Figure 5(c)). In the presence of ouabain there was no difference between the areas under curves in the presence of 1 mM and 2.5 mM [Ca²⁺]_{out} at any [K⁺]_{out}. Importantly, the effects of ouabain on the sensitivity to ACh and the maximal relaxations were independent of [Ca²⁺]_{out} (Figures 5(d) and 5(e)). However, ouabain suppressed the maximal relaxations at 4.2 mM [K⁺]_{out} more than in the presence of 5.9 mM [K⁺]_{out} (Figure 5(e)).

To assess the importance of K_{ir} for the effect of [Ca²⁺]_{out} we repeated the experiments in the presence of 30 μM BaCl₂. BaCl₂ inhibited the ACh-dependent relaxation only when the bath solution contained 4.2 mM [K⁺]_{out} (Figure 6(a)). In the presence of BaCl₂ no differences between the areas under the curve at 1 mM and 2.5 mM [Ca²⁺]_{out} were seen (Figure 6(c)). No effect of BaCl₂ on the relaxations at 5.9 mM [K⁺]_{out} was seen.

The effect of BaCl₂ at 4.2 mM [K⁺]_{out} was dependent on [Ca²⁺]_{out}. BaCl₂ suppressed the sensitivity to ACh only when [Ca²⁺]_{out} was 2.5 mM (Figure 6(d)). In contrast, the maximal relaxations were similarly suppressed in the presence of both 1 mM and 2.5 mM [Ca²⁺]_{out} (Figure 6(e)).

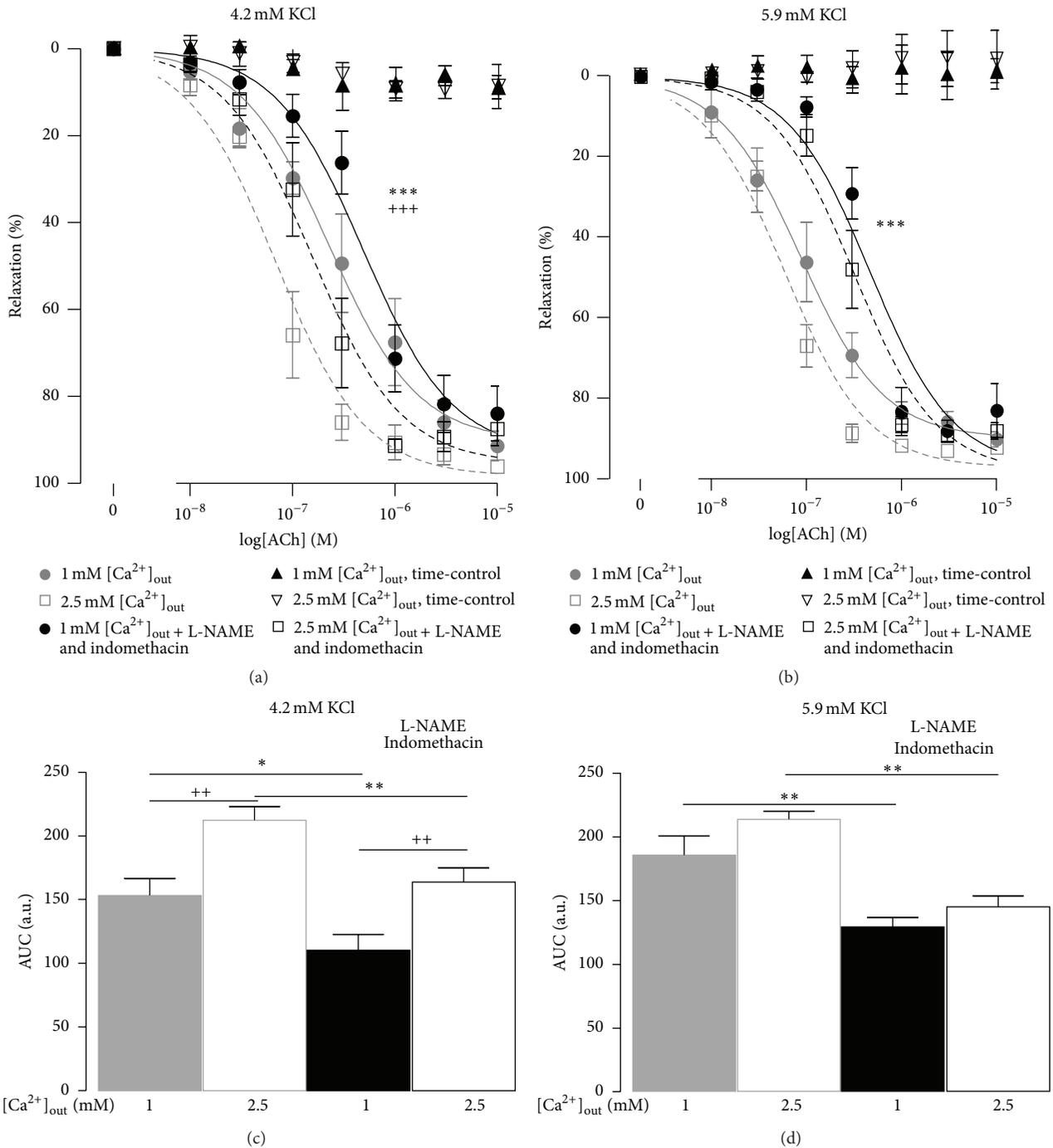


FIGURE 2: The concentration-dependent relaxation to ACh in the bath solutions containing either 4.2 mM ((a) $n = 8$) or 5.9 mM ((b) $n = 6$) $[K^+]_{out}$. In the time-control experiments only the vehicle was supplied ($n = 2-4$). Responses were compared under control conditions and after preincubation with $100 \mu\text{M}$ L-NAME and $3 \mu\text{M}$ indomethacin. (c) and (d) show the areas under curve (AUC) for the concentration-responses shown in (a) and (b). ++ and +++ indicate $P < 0.01$ and 0.001 for responses in the presence of 1 mM $[Ca^{2+}]_{out}$ versus 2.5 mM $[Ca^{2+}]_{out}$. ** and *** indicate $P < 0.01$ and 0.001 for responses before and after application of L-NAME and indomethacin.

4. Discussion

We have studied the $[Ca^{2+}]_{out}$ -dependent modulation of EDH-like signaling and the role of IK_{Ca} and the $[K^+]_{out}$ sensors, that is, the Na^+, K^+ -ATPase and K_{ir} channels, for this modulation.

4.1. $[Ca^{2+}]_{out}$ and the IK_{Ca} Channels. We found that an elevation of $[Ca^{2+}]_{out}$ from 1 to 2.5 mM increases the relaxation to ACh. This was also seen after the blockade of NO- and prostaglandin-dependent pathways. This is in accordance with the previous observations where wall tension

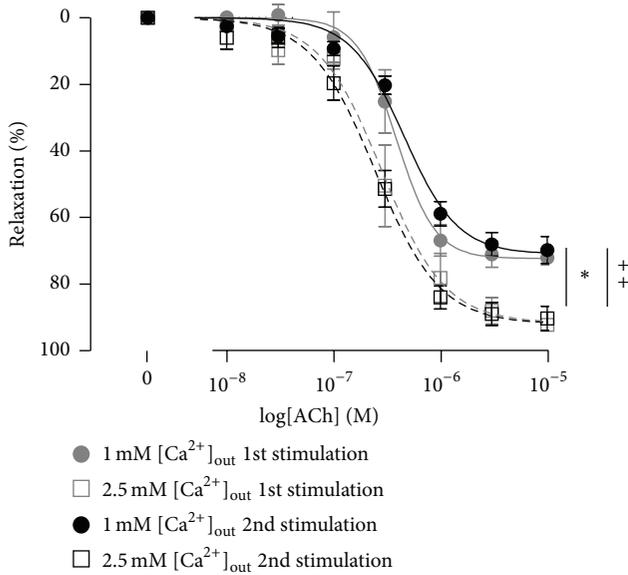


FIGURE 3: Repeated ACh concentration-relaxation curves in the presence of 100 μ M L-NAME and 3 μ M indomethacin ($n = 5$). Relaxations were first performed in the presence of either 1 mM or 2.5 mM $[Ca^{2+}]_{out}$; the bath solution was then replaced with either 2.5 mM or 1 mM $[Ca^{2+}]_{out}$. This protocol was repeated once more. * indicates $P < 0.05$ for 1 mM versus 2.5 mM $[Ca^{2+}]_{out}$ for the 1st stimulations; ++ indicates $P < 0.05$ for 1 mM versus 2.5 mM $[Ca^{2+}]_{out}$ for the 2nd stimulations.

and membrane potential were measured [11]. We did not measure membrane potential, but the sensitivity of NO- and prostaglandin-independent relaxation to TRAM34 and apamin suggests that this effect was mediated via an EDH-like pathway. The effects of two relatively low (1 mM) and high (2.5 mM) $[Ca^{2+}]_{out}$ concentrations were compared, because IK_{Ca} channel-dependent relaxation has previously been shown to differ strikingly at these two concentrations of Ca^{2+} [11].

Although the high $[Ca^{2+}]_{in}$ in activated endothelial cells activates both SK_{Ca} and IK_{Ca} channels, the relative importance of SK_{Ca} and IK_{Ca} has been suggested to depend on $[Ca^{2+}]_{out}$ in the myoendothelial space [7, 8]. It has been suggested that $[Ca^{2+}]_{out}$ is sensed by the G-protein-coupled CaSR [18] which can modulate the IK_{Ca} channel-dependent EDH in the vascular wall [11, 16, 19]. It was therefore tempting to speculate that the effect of $[Ca^{2+}]_{out}$ changes seen in the present study could be explained by changes in the IK_{Ca} activity. However, after inhibition of IK_{Ca} with TRAM34 there was still a modulating effect of $[Ca^{2+}]_{out}$. This suggests that the modulatory role of $[Ca^{2+}]_{out}$ cannot be limited to an effect on the IK_{Ca} channels.

Part of the Ca^{2+} effect may, however, be via the IK_{Ca} channels since TRAM34 affected the ACh concentration-response curves differently at the two $[Ca^{2+}]_{out}$ concentrations. This contrasts with a previous report [11] where TRAM34 eliminated the modulating effect of $[Ca^{2+}]_{out}$ on ACh relaxations. However, the actual values obtained in the previous study [11] were rather similar to those found in

the present study; in the presence of TRAM34 the maximal relaxation tended to be less with 1 mM compared to 2.5 mM $[Ca^{2+}]_{out}$ [11]. Thus, the effect of $[Ca^{2+}]_{out}$ -CaSR signaling on the IK_{Ca} channels likely explains a part of the effect of $[Ca^{2+}]_{out}$ on EDH, possibly via modulation of $[Ca^{2+}]_{in}$ [20, 21].

4.2. $[Ca^{2+}]_{out}$ and the Na^+, K^+ -ATPase/ K_{ir} Channels. Simultaneous inhibition of SK_{Ca} channels with apamin and IK_{Ca} channels with TRAM34 resulted in complete inhibition of endothelium-dependent relaxation. In contrast to TRAM34, the inhibitory effect of apamin was larger at 2.5 mM $[Ca^{2+}]_{out}$. It is therefore possible that $[Ca^{2+}]_{out}$ may also modify the activity of the SK_{Ca} channels. Another possibility is that $[Ca^{2+}]_{out}$ has an effect downstream for activation of the SK_{Ca}/IK_{Ca} channels.

A downstream effect of $[Ca^{2+}]_{out}$ might include an effect on the two K^+ sensors, the ouabain-sensitive Na^+, K^+ -ATPase and K_{ir} channels, which are also known to be involved in EDH signaling [5]. To address this possibility we assessed the effect of inhibition of the Na^+, K^+ -ATPase with ouabain [11, 22] and the effect of inhibition of the K_{ir} channels with Ba^{2+} [23, 24]. Both these interventions reduced the relaxation to ACh as expected. Importantly, in the presence of both ouabain and Ba^{2+} $[Ca^{2+}]_{out}$ had no effect on the ACh response. This might suggest that the effect of $[Ca^{2+}]_{out}$ is mediated via the Na, K -ATPase and K_{ir} channels.

Interestingly, inhibition of either the Na^+, K^+ -ATPase or K_{ir} channels caused more than 50% inhibition of the relaxation at 4.2 mM $[K^+]_{out}$. If these K^+ sensors act strictly in parallel, this is surprising. A possibility is that these transporters interact functionally and support the activity of each other. Thus, it has previously been shown that the Na^+, K^+ -ATPase associates functionally with some K^+ channels, for example, the ATP-dependent K^+ channels (K_{ATP}) [25]. K^+ ions leaving the cell through the K^+ channels have been shown to supply the Na^+, K^+ -ATPase with K^+ even in $[K^+]_{out}$ -free media [26], while the Na^+, K^+ -ATPase activity provides a gradient for the ionic current through the K^+ channels [25]. A similar functional interaction has previously been reported in the heart [27] and pancreas [28]. It is possible that the Na^+, K^+ -ATPase and the K_{ir} channels may also interact functionally to short-circuit the membrane K^+ transport.

Both the Na^+, K^+ -ATPase and K_{ir} channels are expressed in the smooth muscle cells, although they may also be present in the endothelial cells [11, 19, 29]. The effect of $[Ca^{2+}]_{out}$ might therefore be mediated via either smooth muscle or endothelial cells. Some studies reported that the effect of Ba^{2+} is endothelium-dependent [14, 30–33]. These endothelial K_{ir} channels can still be modulated by a “ K^+ cloud” thereby amplifying endothelial hyperpolarization which then spreads through the myoendothelial gap junctions. The present study was performed on endothelium-intact arteries making it impossible to distinguish the functional localization of the Na^+, K^+ -ATPase and K_{ir} channels. But regardless of their localization our results indicate that the Na^+, K^+ -ATPase or K_{ir} channels or both are modulated by $[Ca^{2+}]_{out}$ and that they act as sensors for $[K^+]_{out}$. The localized $[K^+]_{out}$ can act either

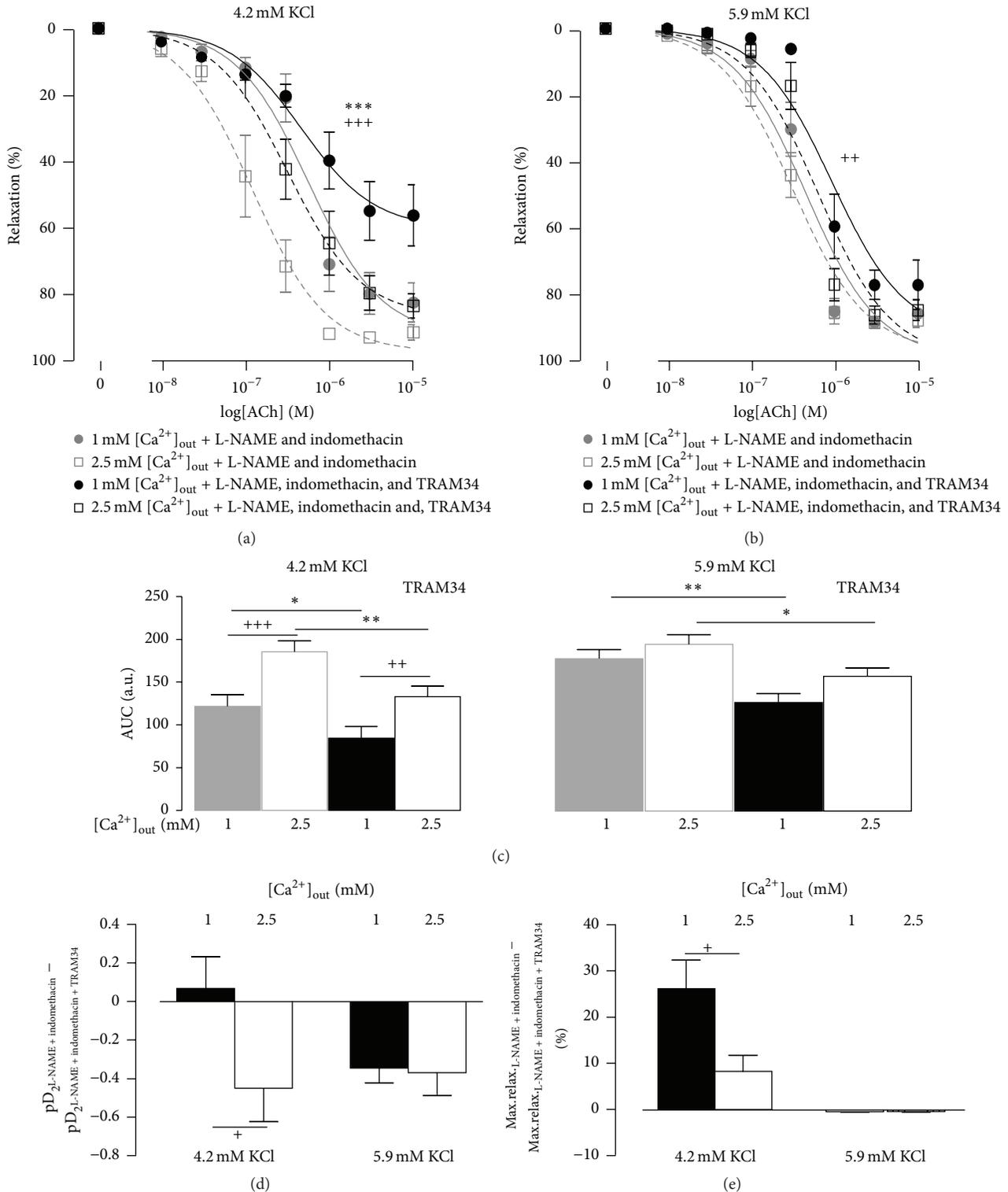


FIGURE 4: The concentration-dependent relaxation to ACh in the bath solutions containing 4.2 mM ((a) $n = 8$) and 5.9 mM ((b) $n = 8$) $[K^+]_{out}$. Responses were compared after preincubation with 100 μ M L-NAME and 3 μ M indomethacin (grey curves) and after preincubation with 100 μ M L-NAME, 3 μ M indomethacin, and 1 μ M TRAM34 (black curves). (c) shows the areas under curve (AUC) for the concentration-response curves shown in (a) and (b). (d) shows a shift in sensitivities to ACh (pD_2) after addition of TRAM34 in the experiments shown in (a) and (b). (e) shows changes in the maximal relaxations (to 10 μ M ACh) for the experiments shown in (a) and (b). +, ++, and +++ indicate $P < 0.05$, 0.01, and 0.001 for responses in the presence of 1 mM versus 2.5 mM $[Ca^{2+}]_{out}$. *, **, and *** indicate $P < 0.05$, 0.01, and 0.001 for responses before and after incubation with TRAM34.

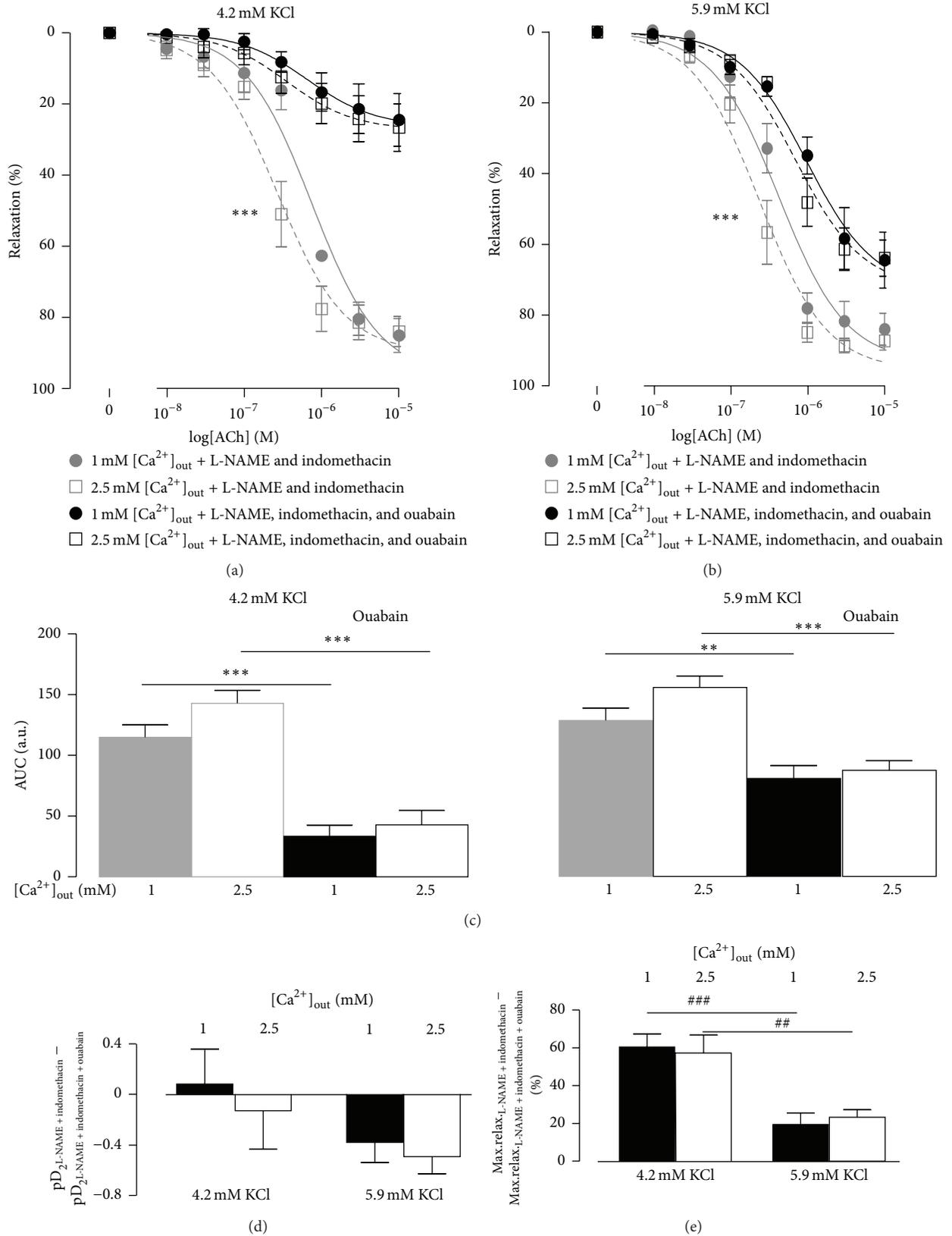


FIGURE 5: Incubation with 10 μ M ouabain in the presence of 100 μ M L-NAME and 3 μ M indomethacin suppressed ACh-dependent relaxations. The concentration-response curves were constructed for the experiments in the bath solutions containing 4.2 mM ((a) $n = 8$) and 5.9 mM ((b) $n = 8$) $[K^+]_{out}$. (c) shows the areas under curves (AUC) from (a) and (b). The changes in sensitivities to ACh (pD_2) before and after incubation are shown in (d). The changes in maximal relaxations (to 10 μ M ACh) before and after the treatment with ouabain are shown in (e). ** and *** indicate $P < 0.01$ and 0.001 for the responses before and after incubation with ouabain. ## and ### indicate $P < 0.01$ and 0.001 for 4.2 mM versus 5.9 mM $[K^+]_{out}$.

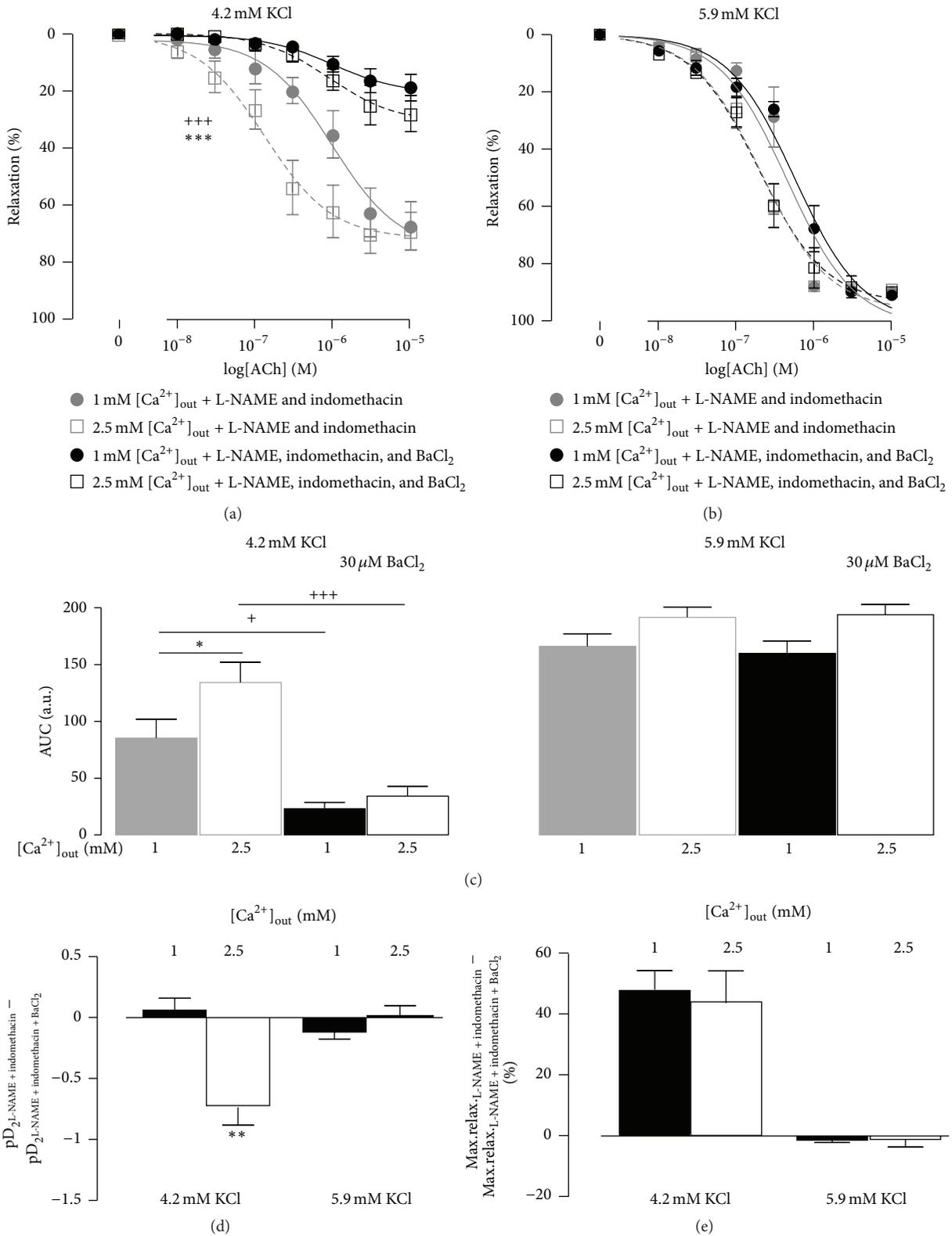


FIGURE 6: Addition of $30 \mu M BaCl_2$ to the solution containing $100 \mu M$ L-NAME and $3 \mu M$ indomethacin suppressed ACh-dependent relaxations. The concentration-response curves were constructed in the bath solutions with 4.2 mM ((a) $n = 6$) and with 5.9 mM ((b) $n = 6$) $[K^+]_{out}$. (c) shows the areas under curves (AUC) as in (a) and (b). Changes in the sensitivities to ACh (pD_2) after addition of $BaCl_2$ are shown in (d). The effects of $BaCl_2$ on the maximal relaxations are shown in (e). ** indicates $P < 0.01$ versus 1 mM $[Ca^{2+}]_{out}$.

as EDHF (in case of smooth muscle cell localization of the K^+ sensors) or by amplifying the endothelial hyperpolarization which then spreads through the myoendothelial gap junctions (in case of endothelial localization of the K^+ sensors).

4.3. The Localized $[K^+]_{out}$ Signaling. The importance of $[Ca^{2+}]_{out}$ was studied at two concentrations of $[K^+]_{out}$. We chose values close to physiological values for rats based on the observation that increase in $[K^+]_{out}$ to 5.9 mM induces $BaCl_2$ -sensitive relaxation while relaxation to $[K^+]_{out}$ above 5.9 mM is diminishing [13, 19, 30, 34]. In addition, it has been suggested that the Na^+, K^+ -ATPase in the vasculature is fully saturated at 5.9 mM $[K^+]_{out}$ [24, 35]. This suggests that any relaxation in the presence of 5.9 mM $[K^+]_{out}$ is unlikely to be caused by an increase of $[K^+]_{out}$.

This suggestion was supported by our observation that in the presence of 5.9 mM $[K^+]_{out}$ ouabain and particularly Ba^{2+} had a little effect on the relaxation, while at 4.2 mM $[K^+]_{out}$ pronounced effects of these inhibitors were seen. This could be because the elevation of $[K^+]_{out}$ to 5.9 mM saturates the Na^+, K^+ -ATPase and K_{ir} channels in agonist precontracted arteries [19]. In fact, the “ K^+ cloud” can be generated not only by the endothelium but also via K^+ efflux from big-conductance Ca^{2+} -activated K^+ channels which are activated in depolarized smooth muscle cells of agonist precontracted arteries [14]. It has previously been shown that, under experimental conditions where the K^+ -sensors are saturated, inhibition of the Na, K -pump and K_{ir} channels has no effect on relaxation [14]. This indicates that the EDH under these conditions spreads through other mechanisms, possibly as a hyperpolarizing current via myoendothelial gap junctions.

Consistent with this, the modulating effect of $[Ca^{2+}]_{out}$ seen at 4.2 mM $[K^+]_{out}$ was abolished at 5.9 mM $[K^+]_{out}$. This finding is further consistent with our suggestion that the modulatory function of $[Ca^{2+}]_{out}$ is mediated largely via the Na, K -pump and K_{ir} channels. Finally, the lack of effect of TRAM34 at 5.9 mM $[K^+]_{out}$ is consistent with the hypothesis [11] that the importance of the IK_{Ca} channels for the EDH is associated with a localized increase in $[K^+]_{out}$ [12] which acts through the Na, K -pump and K_{ir} channels.

While no effect of Ba^{2+} was seen at 5.9 mM $[K^+]_{out}$, a small but significant inhibition of the relaxation was observed in the presence of ouabain. If at this $[K^+]_{out}$ the Na^+, K^+ -ATPase is completely saturated as suggested [24, 35], it is possible that the observed effect of ouabain was mediated via modulation of gap junctions. It has previously been shown that inhibition of the Na^+, K^+ -ATPase suppresses intercellular communications, including myoendothelial gap junctions [22, 26, 36, 37]. Thus, part of the ouabain effect can be related to inhibition of EDH spreading to smooth muscles through the myoendothelial gap junctions.

5. Conclusion

The main findings of this study are as follows: (i) an elevation of $[Ca^{2+}]_{out}$ enhances the EDH-like relaxation to ACh, but (ii) this Ca^{2+} effect disappears with an elevation of $[K^+]_{out}$.

(iii) The effect of $[Ca^{2+}]_{out}$ is maintained after blocking the IK_{Ca} channels, but (iv) it disappears after blockade of the Na^+, K^+ -ATPase and K_{ir} channels. (v) Finally, inhibitors of the Na^+, K^+ -ATPase and K_{ir} channels, ouabain and Ba^{2+} , have large effect on EDH-like relaxation only when $[K^+]_{out}$ is low. Thus, we have suggested that the localized $[K^+]_{out}$ signaling acts through the Na^+, K^+ -ATPase and K_{ir} channels, and we have provided strong evidence that these two K^+ sensors are affected by $[Ca^{2+}]_{out}$.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Christian Aalkjaer and Vladimir V. Matchkov contributed equally to this paper.

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